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The Practical and Fundamental Limits of Optical Imaging in Mammalian Brains

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Advances in chemistry and physics have profound effects on neuroimaging. Current and future progress in these disciplines will continue to aid in efforts to visualize neural circuitry, particularly in deeper layers of the brain.

In "Histology of the Nervous System of Man and Vertebrate," Santiago Ramón y Cajal stated that "the key to understanding turns on the nature of available instrumentation" and that "current methods and ideas are entirely dependent on continuing progress in chemistry and physics, which remain the principle allies of the naturalist" (Ramon y Cajal, 1995). He would know; although optical microscopy was first used to observe biological samples more than 200 years before his time, only with the emergence of high-quality commercial microscopes and Cajal's own improvement upon Camillo Golgi's silver chromate method, did he make the series of discoveries that led to the neuron doctrine and the beginning of modern neuroscience.

Chemistry and physics have gone through paradigm-shifting changes of their own in the more than 100 years since Cajal's declaration, but their roles in neuroscience have remained the same: they provide the foundation for and set the limits on the methods with which questions in neuroscience are answered and understanding is reached. A perfect example is two-photon fluorescence microscopy (TPFM), a powerful technique for imaging structures in living tissues. Since its invention 25 years ago (Denk et al., 1990), TPFM has become an essential tool for structural and functional imaging of neurons at subcellular spatial resolution in scattering brains. Here we examine it in relationship to physics and chemistry, for they help us understand the limitations and the future directions of not only TPFM, but also other imaging modalities.

Fluorescence generation occurs when a molecule returns from an excited electronic state to its ground electronic state by emitting a photon. The molecule may reach the excited state by absorbing a photon at a particular wavelength, or by absorbing two photons of a much longer wavelength simultaneously. The probability of two-photon absorption, however, is exceedingly small and thus requires high-intensity excitation light. Mode-locked near-infrared (0.7–1.1 μm, NIR) lasers with short pulse widths have the peak power necessary for two-photon fluorescence generation for most visibly fluorescent dyes. Experimentally, the energy carried by these short pulses is concentrated by a high-numerical-aperture (NA) microscope objective to a small focal volume, within which the light intensity is high enough for effective two-photon fluorescence generation. This confinement of fluorescence emission to a focus reduces photo-induced bleaching and damage that would otherwise occur with confocal or widefield microscopy (Denk and Svoboda, 1997). Typically, the laser focus is scanned across the sample point by point, and the fluorescence collected and measured at each point is used to build up the image. The resolution of a TPFM is determined by the size of the focus. At moderately high NA (e.g., 0.8), a TPFM can resolve subcellular features such as dendritic spines.

The most technically challenging part of building a TPFM is the light source. After the emergence of commercial ultrafast lasers in the 1990s, both homebuilt and commercial two-photon fluorescent microscopes started to populate laboratories worldwide. One of the main reasons for the popularity of TPFM, particularly in neuroscience, is that it allows 3D imaging in scattering tissues such as live mammalian brains (Svoboda and Yasuda, 2006). Because the fluorescence is only generated at the focus, all the fluorescent photons, scattered or not by the tissue, constitute a useful signal that reflects the sample fluorescent properties at the focal position. In contrast, widefield microscopy techniques (e.g., light-sheet microscopy [Ahrens et al., 2013]) require minimal scattering of the fluorescent signal to form a sharp image, and thus are limited to transparent samples.

What ultimately made TPFM as powerful as it is now is the progress in chemistry, specifically, the developments of versatile fluorescent reagents that can selectively stain the structures of interest and even report the occurrence of physiological events. In the brain, genetic and synthetic fluorescent reporters are now routinely employed in laboratories to monitor morphological and functional changes (Helmchen et al., 2011). For example, recently developed genetically encoded calcium sensors (Chen et al., 2013) have single action potential sensitivity and, when combined with TPFM, allow neuronal activities to be monitored at synaptic, cellular, and network level in the brains of awake, behaving animals (Grienberger and Konnerth, 2012).

To completely map the morphology and function of the brain in a live animal, one needs to be able to image neurons throughout the brain, preferably at the same subcellular resolution that provides synaptic-level information. However, most in vivo studies using TPFM in the scattering mammalian brains are limited to the first few hundred microns, which prevents us from understanding the anatomical and functional inner workings of even the most superficially located

cerebral cortex. Two main physical factors that limit imaging depth and optical manipulation (e.g., optogenetics) are optical aberration and attenuation.

Aberration is familiar to anyone who has gazed out of a rain-drenched window. Optically, light rays on their way to a focus get distorted by structures with different indices of refraction. Instead of converging to the same spot and interfering constructively there, they form an enlarged focus with reduced intensity. The same thing happens when the excitation laser of TPFM is focused through a cranial window and into the brain using a water-dipping objective: both the window and the brain itself aberrate the laser beam and lead to an enlarged, weaker focus, reducing both the resolution and the signal of TPFM. The sample-induced aberration increases with increased imaging depth, leading to the common observation that the image becomes less "sharp." Such aberration is not limited to mammalian brains or TPFM: even relatively transparent samples such as zebrafish and *C. elegans* aberrate light, often significantly, in widefield methods. Aberrations experienced by fluorescence photons when they traverse the sample before reaching the detection camera lead to the loss of signal and image fidelity.

Aberration, at least conceptually, is an easy problem to solve. If we can modify the wavefront of the light rays with a deformable mirror to exactly balance and cancel out the sample-induced aberrations as the light travels to the focus, diffraction-limited, ideal imaging quality can be recovered. These adaptive optics (AO) techniques were first proposed for ground-based telescopes to observe celestial objects through Earth's turbulent atmosphere. In direct analogy to microscopy, this turbulence distorts the light emitted by the celestial object of interest and leads to a blurry image with temporally varying intensity and position (e.g., "twinkle, twinkle, little star"). This distortion can be directly measured with a wavefront sensor and the information used to control the surface of a deformable mirror to cancel it out. Sometimes, when the light from the star itself is too faint, an artificial guide star may be generated by using a laser to excite the sodium atoms in the upper atmosphere. Spectacularly successful in astronomy, the astronomical AO technique can be directly adapted to correct the aberration of transparent tissue. In one example (Wang et al., 2014b), the artificial guide star was generated by two-photon excitation of fluorescent proteins in zebrafish brain, and neuronal processes, unresolved without AO, became distinct after correction. At depth in mammalian brains, however, the light from an induced fluorescent guide star is too highly scattered to be used to measure the wavefront distortion directly (but see below). To address this problem, we have developed several methods (Ji et al., 2010; Wang et al., 2014a), where, by manipulating intensity or phase of light falling on different parts of the microscope objective back aperture, we can measure the aberration associated with strongly scattering samples such as the mouse brain. Unlike atmospheric turbulence that changes on millisecond scale, the brain-induced aberration is generally stable over hours, and the same correction can improve image quality over hundreds of microns (Ji et al., 2012). It is thus well suited for in vivo brain imaging, where large field of view and extended imaging duration are often needed. With AO, we can routinely resolve basal dendritic spines of L5 pyramidal neurons and functionally image axonal boutons in L4 in mouse cerebral cortex in vivo.

A more difficult problem that limits imaging depth is light attenuation caused by absorption and scattering, both of which reduce intensity exponentially with depth. In the brain, hemoglobin absorbs blue and green light strongly, but not red or NIR light (this is why blood appears red to our eye). At the other end, for wavelengths beyond 1 μm, water becomes the dominant absorber. Within this "optical window" (650–1,200 nm), where the light used for two-photon excitation falls, the major source of its attenuation in brain tissue is not absorption, but scattering.

Scattering is also a familiar phenomenon in our daily life: anything that is not visually transparent scatters visible light (e.g., fog, clouds, milk, skin, mouse brain). In the mammalian brain, cytosol intermixes with many lipid-rich structures (e.g., myelin sheath, mitochondria, and endoplasmic reticulum), which with sizes close to the wavelengths of visible and NIR light, dominate the scattering process. Therefore, for TPFM, both the excitation (usually in NIR) and emission (usually in visible) light are attenuated by scattering. Fluorescence emitted in the blue-to-yellow band is additionally absorbed by hemoglobin and some metabolites. To reach a high focal intensity for two-photon excitation, one has to increase the laser power exponentially with depth. Eventually, the intensity at the surface of the tissue is sufficient to excite superficial fluorescent structures without needing the confinement at the laser focus. When this out-of-focus background overwhelms the in-focus signal, further increase of the excitation intensity does not improve image quality, and the imaging depth limit of a standard TPFM is reached (Theer et al., 2003) (in live samples, photo-induced damages may happen well before this limit is reached). Correcting sample-induced aberration could extend the imaging depth limit by improving the in-focus signal, but scattering still poses the ultimate limit.

Reagents have been developed to improve the transparency of fixed brain tissue by increasing tissue optical homogeneity. The varying refractive index of native brain can be matched by either raising the refractive index of the aqueous fraction of the tissue (Hama et al., 2011) or replacing the lipids with a hydrogel-based infrastructure (Chung et al., 2013). A physiological reagent that can clear living mammalian brains, however, has yet to be found. For now, scattering is here to stay.

For some structures (e.g., hippocampus in mouse), the overlaying tissue may be removed to provide optical access without notably impacting the question under investigation. However, such an approach is not realistic for more deeply buried nuclei (e.g., amygdala, hypothalamus). In such cases, endoscopy is more practical. With a long history of its own (first developed in 1806), endoscopes are miniature imaging devices that may be directly inserted into the organ and are widely used in the medical field for diagnosis and treatment. Microendoscopes based on gradient refractive index (GRIN) lenses or optical fibers have been developed to image the mammalian brain with cellular resolution. The single-photon excitation versions of these instruments are small enough to be mounted on a

freely moving animal (e.g., Ghosh et al., 2011). Its widefield detection modality makes it insensitive to brain motion, but with the concomitant disadvantage of having no optical sectioning capability. Two-photon microendoscopy allows high-resolution volume imaging of neurons, but is sensitive to brain motion, since it is slower and produces images serially in time. It is thus better suited to head-fixed animals. Commercially available GRIN lenses as small as 0.5 mm in diameter can provide an imaging field of view of 0.2 × 0.2 mm^2 at single-neuron resolution. While future improvements of these devices can be expected, we have also improved their performance by correcting the aberrations induced by the GRIN lens itself with AO.

Even without clearing reagents or tissue removal, scattering is not insurmountable. Just as wavefront shaping can cancel out the optical aberration, the wavefront of the excitation can be manipulated on a much finer scale to partially compensate for scattering. Sharp images have been generated with large signal gain from light that has traveled many scattering lengths through tissue (e.g., Tang et al., 2012; Yaqoob et al., 2008). However, because the scattering profile of biological samples varies rapidly in space and often also in time, to correct scattering in a brain volume hundreds of microns in dimension, many thousands or millions of corrective wavefronts would need to be measured and applied at speeds far beyond the capabilities of current methods and instrumentation. Therefore, except for niche applications where micron-sized fields of view are sufficient, the microendoscopy approach remains the most accessible and robust imaging method for deeply buried nuclei in the mammalian brain.

Despite the significant role that physics has played in improving neuroimaging as described above, the advances arising from chemistry have remained the most accessible, because reagents are generally inexpensive and simple to disseminate. As mentioned above, both tissue scattering and sample-induced aberration are weaker at longer wavelengths, and absorption is smallest in the far-red to NIR window. Hence, deeper imaging may be reached simply by employing red-shifted fluorescent dyes (Kobat et al., 2009). Indeed, with even longer wavelengths for three-photon excitation, subcortical structures in mouse brain have been imaged in vivo (Horton et al., 2013). A red genetically encoded calcium indicator, when combined with the continuing improvements in optics (e.g., AO, better excitation light sources in NIR, and objectives designed to maximize the collection of scattered photons), may make functionally imaging the whole mouse cortex a reality in the near future.

The longer wavelengths of both the excitation and fluorescence photons of deep red or IR reagents make them less scattered and absorbed than their shorter-wavelength counterparts. Hence, widefield detection methods that so far have been limited to visibly transparent samples by scattering, such as structured illumination or light-sheet microscopy, may become viable alternatives to TPFM in certain applications in the scattering brains. With their fast parallel readout, these methods are more compatible with reagents that rely on rapid contrast mechanisms (e.g., voltage-sensitive dyes). For AO, my lab has also used fluorescence of the red-shifted dyes as a guide star for direct wavefront measurement and correction of aberration in the mouse brain at 700 μm depth, something not possible with traditional green/yellow-emitting dyes.

No matter how clever the physical principle, the power of every imaging method is confined by the practically achievable signal-to-noise ratio, which is often limited by the brightness of the dye—the usefulness of many fast imaging techniques is currently limited by the need to average the data in time to achieve sufficient signal. Regardless of the color of the fluorescence, a real game changer for all imaging endeavors would be a fluorescent reagent 10^4 × (or even 100 ×) brighter than the existing ones. Before such a magic dye is found, however, with the currently available fluorescent probes and standard microscopes, there are things we can do to make life easier. First, be mindful about aberration. Use sample preparation protocols that introduce minimal aberration (i.e., anything with a refractive index different from that of the immersion media of the objective). Minimize brain-induced aberration by positioning the brain as perpendicular to the optical axis of the microscope objective as possible (otherwise, the correction collar of the microscope objective is of little help). Be smart about labeling strategy. When imaging neurons at depth, only label the neurons of interest—reducing fluorescence elsewhere delays the appearance of the out-of-focus background when increasing the excitation power. Finally, a word of caution: the act of observation itself may alter the state of the system. Although often billed as noninvasive, optical imaging is no exception when it comes to the potential for unintended consequences, caused by introducing tens to hundreds of milliwatts of light and millions to billions of fluorescent molecules into each neuron. Each system has a practical photon budget, i.e., the maximal amount of photons extractable before photon- or probe-induced artifacts make the system non-physiological. Spend it wisely.

It seems fitting to end this essay with Cajal, who predicted back before the quantum revolution that "in the distant future, when science has developed vast resources and chemistry and physics are no longer regarded as separate ways of approaching the same mechanisms at the level of atoms, anatomy will be an even more rigorous discipline," where questions on function, mechanism, and development of the brain may finally be answered. That distant future has become a reality, and it is now up to us to use the, albeit still imperfect, tools developed in physics and chemistry wisely to answer the fundamental questions of how the brain works.

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